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16. ABSTRACT

The present study was conducted to evaluate the mutagenicity of a domestic wastewater and a combined domestic/industrial wastewater at various stages of conventional treatment. Influent (raw wastewater) and effluent samples were subjected to sequential acid/base liquid-liquid extractions with methylene chloride and the extracts were assayed for mutagenic activity in the Ames test. S. typhimurium strain TA98 gave the highest responses of the tester strains to mutagens present (up to approximately 20,000 revertants/liter), but activity was also detectable with strains TA1537 and TA100. The majority of the activity was direct-acting but in some cases significant enhancement of the activity was seen after activation with a rat liver S9 fraction. Mutagen levels based on TA98 activity in the industrial/domestic effluents were 7 to 45-fold higher than in the domestic effluent. Despite the consistent efficient removal (> 90%) of extractable organics by activated sludge treatment, the removal of compounds with mutagenic activity was relatively poor and highly variable as evidenced by increases of 4 to 20-fold in the specific mutagenicities of the extracts on a per mg basis. Chlorination at the secondary effluent stage had little influence on the mutagenicity seen.

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JOURNAL ARTICLE

Evaluation of conventional treatment processes for removal of mutagenic activity from municipal wastewaters

John R. Meier, Dolloff F. Bishop

The discharge of toxic organic compounds into our public water supplies has become an important and growing environmental concern, both with respect to direct impacts on the ecology of receiving bodies of water and with regard to potential effects on human health. A number of studies have demonstrated mutagenic activity in municipal wastewater effluents.[1-4] The concern over exposure to mutagenic agents in our environment is primarily based on the premise that chemicals which damage deoxyribonucleic acid (DNA) in germ cells may increase the incidence of heritable mutation and genetic disorders, and chemicals that damage DNA in somatic cells may result in the initiation of cancer. The latter concern has received considerable attention because of reports from several studies indicating a high qualitative correlation in the ability of chemicals to induce cell mutation in bacteria and to cause cancer in laboratory animals.[5-7] The possibility that exposure to mutagenic agents may also play a role in the etiology of other human diseases including atherosclerosis, senile cataracts, and various metaplasias has also been proposed.[8]

Previous studies on the mutagenicity of municipal wastewater have focused primarily on advanced treatment processes for reuse.[1-3] This study was undertaken to determine the ability of conventional treatment processes to remove mutagenicity from municipal wastewaters. A previous study conducted at the Environmental Protection Agency (EPA) Mill Creek Test and Evaluation (T&E) Facility in Cincinnati, Ohio, by Petrasek *et al.*[9] demonstrated greater than 90% removal for most of 22 priority pollutant organic compounds, using a pilot-scale conventional primary/activated sludge secondary process. It was interesting to see whether a similar reduction in mutagenic activity could be achieved using the same treatment sequence at the T&E facility. In addition, two full-scale treatment plants, one receiving a heavily industrialized municipal waste and the other a primarily domestic waste, were also examined for mutagen removal at different stages of treatment.

METHODS

Twenty-four-hour manually flow-proportioned composite samples of raw wastewater, primary effluent, and secondary effluent were collected at the Mill Creek and at the Muddy Creek Treatment Plants in Cincinnati, Ohio, and at the EPA T&E Facility (located adjacent to the Mill Creek Plant). The Mill Creek Plant receives raw wastewater that is heavily impacted by industrial discharge; the Muddy Creek Plant processes wastewater that is primarily domestic. A typical schematic of the conventional treatment processes for the two plants and the T&E Pilot Plant is shown in Figure 1. A batch chlorination step (10 mg/L Cl_2) was applied to the secondary effluent only at the T&E Facility.

The wastewater composite samples were acidified to pH 2 with 12N HCl and stored at 4°C to prevent biological activity prior to extraction. The procedure used for extraction is shown in Figure 2. The continuous liquid/liquid extraction approach (EPA Test Method 625)[10] using methylene chloride was modified in this study to first extract acid and neutral compounds at pH 2 and then to extract bases at pH 11. The extraction of acids/neutrals from 2-L aliquots of each sample, followed by extraction of bases from the same aliquot conveniently permitted direct use of the stored (pH 2) wastewater samples and minimized any saponification reactions during extraction at pH 11. The acid/neutral and base extracts were combined for the subsequent bioassay tests.

In mixtures as complex as municipal wastewaters, metals and inorganic pollutants can be identified by standard analyses and their effects estimated directly.

A sufficient but variable number of aliquots of each wastewater sample were extracted to supply the amount of extracted organics needed for mutagenicity testing. The extracted organics from each aliquot were partially concentrated by evaporation in a solvent hood. They were then combined, and further concentrated to about 10 mL by Kuderna-Danish procedures.[10] After removal of duplicate aliquots for residue weight determination, the combined extracts were solvent-exchanged with dimethylsulfoxide (DMSO) to eliminate interferences in the mutagenicity bioassay caused by methylene chloride. Additional DMSO was then added to adjust the extracts to a final concentration of 20 mg of residue organics per millilitre of DMSO. The determination of residue organic concentrations, while not a measurement of actual extract concentration of organics (only non-volatile components are measured), permitted a way of establishing standard dose levels in the bioassay. The time between sample collection and final extract preparation averaged about 10 days, depending on the volume of wastewater processed. The extracts were stored at −20°C for up to 2 months until time of assay.

Evaluation of the wastewater extracts for mutagenic activity was performed on coded samples using the histidine reverse mu-

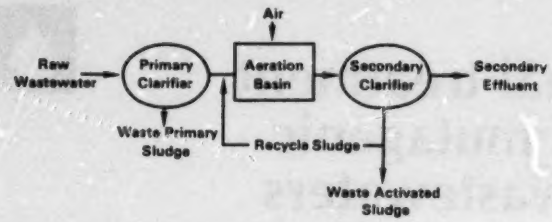

Figure 1—Schematic diagram of conventional wastewater treatment processes.

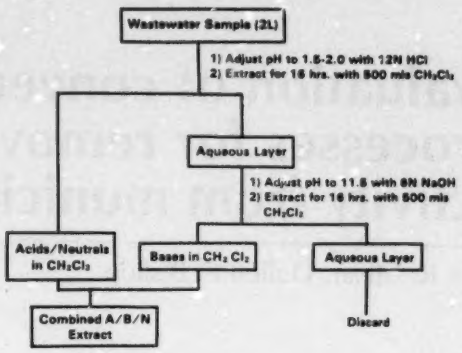
Wastewater Sample (2L)
1) Adjust pH to 1.5-2.0 with 12N HCl
2) Extract for 16 hrs. with 800 mls CH_2Cl_2
Aqueous Layer
1) Adjust pH to 11.5 with 6N NaOH
2) Extract for 16 hrs. with 800 mls CH_2Cl_2
Acids/Neutrals in CH_2Cl_2
Bases in CH_2Cl_2
Aqueous Layer
Combined A/B/N Extract
Discard

Figure 2—Method for extraction of wastewater.

tation assay described by Ames *et al.*[11] Assays were conducted using strains TA98 and TA100, and in some cases also with strains TA1535 and TA1537. For evaluation of mutagens requiring metabolic activation, a liver homogenate fraction (S9) from Aroclor 1254-pretreated rats was added at a level of 50 μL S9 per plate, along with required activation cofactors. Dose-response data were obtained by assaying the extracts at 6 to 8 dose levels of up to 2 mg per plate, using duplicate plates per dose. Lower doses were prepared by two-fold serial dilution of the top dose in DMSO, and plating in a constant volume of 100 μL. Appropriate DMSO solvent and positive control chemicals were included with each assay. Assessment of a positive response was based on a two-fold above background, dose-related response. Estimates of the mutagenic potencies of the extracts were based on the apparent initial slopes of the dose-response curves.

Cytotoxicity assays were performed by plating 300 to 400 TA100 cells on nutrient agar in the absence and presence of the extracts, and by checking for the differential survival of the bacteria. EC_{50} values (effective concentration giving 50% lethality) were calculated by linear regression analysis of the kill curves after a log transformation of the dose.

RESULTS

In the initial studies (T&E Pilot Plant samples, Series A_1 and A_2) the cytotoxicity of the wastewater extracts to the bacteria used in the Ames test was evaluated. The results (Table 1) show that the toxicity of the extracts to *S. typhimurium* TA100 decreased markedly following secondary treatment. The mutagenicity dose-response curves for these initial samples are shown in Figures 3 to 6. Except for one test (Figure 3, A_1, primary effluent), the results consistently showed a substantial increase in the specific mutagenicity of the extracts on a per milligram of residue basis following secondary treatment. The levels of mutagenic activity estimated from the initial slopes of the dose-response curves are presented in Table 2. In contrast to the increase in specific mutagenic activity, there was a substantial decrease in the concentration of extractable residue organics after secondary treatment (Table 2). This pattern was confirmed with three subsequent samplings—Series B_1 and B_2 from the Mill Creek Plant, and Series C from the Muddy Creek Treatment Plant (Table 2).

Mutagenicity was not detectable in many of the extracts from samples taken before secondary treatment, especially for strain TA100. On the other hand, all of the samples showed activity after secondary treatment. This finding seems to be largely at-

Table 1—Toxicity of organic extracts of wastewater samples[a] at different stages of treatment for *S. typhimurium* TA100.

	% Survival[b]							
	Raw wastewater		Primary effluent		Secondary effluent		Secondary effluent + CL_2	
Dose (mg/plate)	A_1[a]	A_2[a]	A_1	A_2	A_1	A_2	A_1	A_2
0.000	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)
0.010	98	99	98	96	114	96	107	110
0.025	98	95	88	105	119	98	103	102
0.050	89	101	82	103	118	101	110	116
0.100	85	97	44	110	121	94	91	102
0.250	41	48	9	38	106	90	83	77
0.500	8	12	0	17	93	49	56	32
1.000	1	2	0	1	37	6	5	2
EC_{50} (mg/plate)[c]	0.16	0.20	0.08	0.21	1.06	0.43	0.42	0.36

[a] Collected at Mill Creek EPA Testing and Evaluation (T&E) facility. A_1 and A_2 refer to samples collected on 9/2/81 and 9/9/81, respectively.

[b] Based on the number of bacteria (out of 300 to 400 cells plated) surviving treatment with the extracts on nutrient agar plates in comparison to untreated controls. Values are averages from duplicate determinations at each dose.

[c] Effective concentration giving 50% lethality.

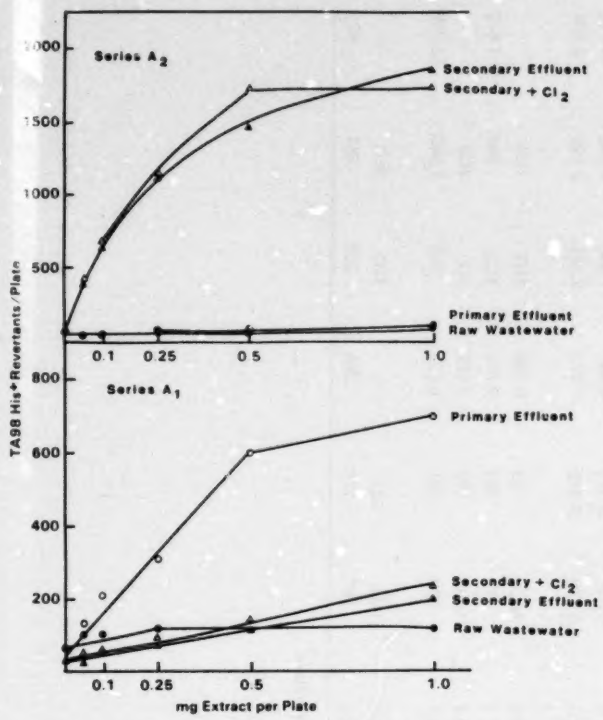

Figure 3—Mutagenic activity of organic extracts of wastewater samples collected at the Mill Creek T&E facility for *S. typhimurium* strain TA98 in the absence of metabolic activation. (Series A_1 and A_2 refer to sample designations used in Table 2.)

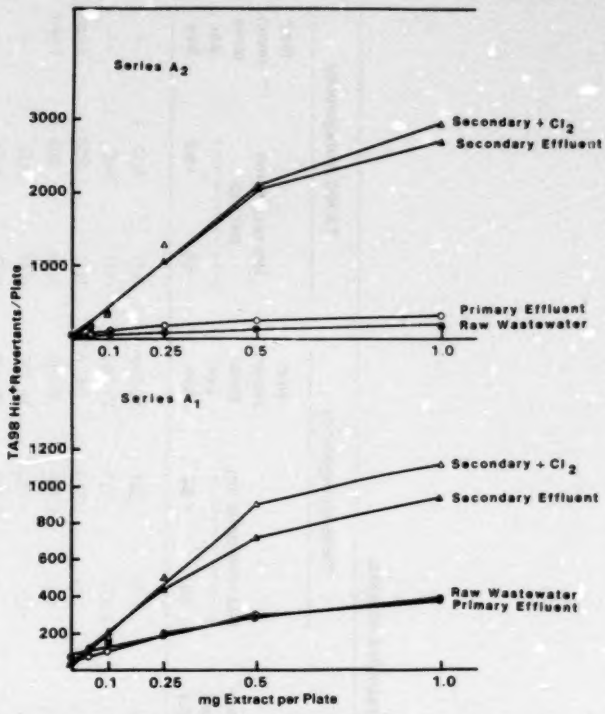

Figure 4—Mutagenic activity of organic extracts of wastewater samples collected at the Mill Creek T&E facility for *S. typhimurium* strain TA98 in the presence of metabolic activation.

tributable to the higher toxicity and lower specific mutagenicity of the extracts from samples taken before versus those after secondary treatment. It should be noted that the original wastewater volumes represented by each milligram of organic residue tested were much higher for the secondary effluent samples than for the raw wastewater and primary effluent samples. The litre-equivalent amounts per milligram of extract ranged from 0.011 to 0.027 for the raw wastewater samples and from 0.115 to 0.455 for the secondary effluents. Because all the extracts were tested over the same dose range in milligram amounts (up to 2 mg per plate), the litre-equivalent amounts tested differed considerably among samples. This aspect of the test procedure undoubtedly influenced the ability to detect activity, although it should have no influence on the level of activity in samples that gave a response. Equal litre amounts of wastewater could not be tested for all samples, especially from raw wastewater, because of high cell-killing and resultant decreasing dose-response curves which were generally evident at dose levels above 1 mg of residue organics per plate. The degree of solubility of the extracts in DMSO would also limit attainable dose levels.

Chlorination of the secondary effluent, which was examined only in the pilot-plant studies, had little influence on either the level of extractable organics or on the specific mutagenicity for strains TA98, but it did seem to significantly increase the TA100 mutagenicity levels.

Because of limiting sample amounts, testing was generally restricted to assays with strains TA98 and TA100, which are generally the most sensitive of the *Salmonella* tester strains to mutagens in water samples. However, for one of the secondary effluent extracts (Series B_1) strains TA1535 and TA1537 were also included in the testing to better examine the strain specificity

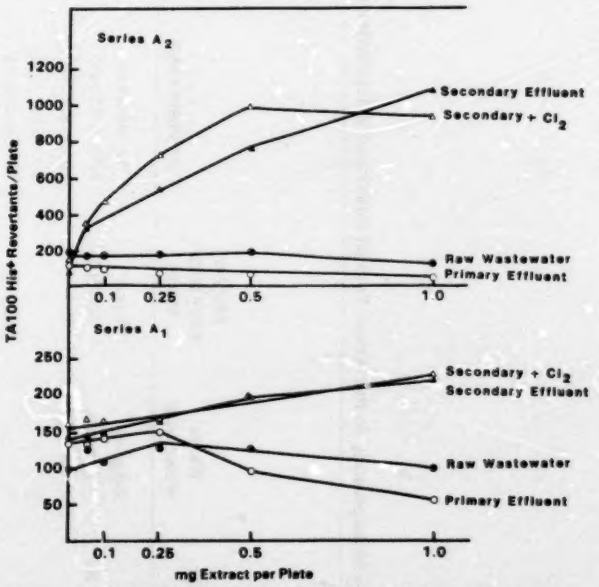

Figure 5—Mutagenic activity of organic extracts of wastewater samples collected at the Mill Creek T&E facility for *S. typhimurium* strain TA100 in the absence of metabolic activation.

Table 2—Mutagenicity in the Ames Test of municipal wastewater samples at various treatment stages.

Treatment plant	Wastewater type	Sample designation (date)	Treatment stage	Total litres extracted	Concentration[a] (mg/L)	TA98 mutagenicity: Net revertants mg −S9	TA98 mutagenicity: Net revertants mg +S9	TA98 mutagenicity: Net[b] revertants per litre	TA100 mutagenicity: Net revertants per mg −S9	TA100 mutagenicity: Net revertants per mg +S9	TA100 mutagenicity: Net[b] revertants per litre
Mill Creek T&E Facility	Industrial/ domestic	Series A_1 (09/02/81)	Raw wastewater	2	48.8	173	381	18 593	N.D.	N.D.	—
			Primary effluent	2	41.8	1 057	524	21 903	N.D.	N.D.	—
			Secondary effluent	6	4.9	169	1 376	6 674	N.D.	258	1 251
			Secondary + Cl_2	6	4.6	196	1 768	8 133	N.D.	409	1 881
		Series A_2 (09/09/81)	Raw wastewater	2	66.5	N.D.	182	12 103	N.D.	N.D.	—
			Primary effluent	2	46.0	77	392	18 032	N.D.	N.D.	—
			Secondary effluent	6	4.6	5 645	4 074	18 944	1 836	1 035	4 813
			Secondary + Cl_2	6	4.0	6 370	4 318	17 272	2 246	2 146	8 584
Mill Creek Plant	Industrial/ domestic	Series B_1 (08/31/82)	Raw wastewater	8[c]	91.0	N.D.	89	8 099	N.D.	N.D.	—
			Secondary effluent	50[c]	7.7	417	353	2 718	N.D.	244	1 879
		Series B_2 (06/20/83)	Primary effluent	23	70.4	N.D.	129	9 082	N.D.	N.D.	—
			Secondary effluent	86	8.7	693	761	6 621	928	1 540	13 398
Muddy Creek Plant	Domestic	Series C (01/11/83)	Raw wastewater	24[c]	36.8	N.D.	ND.	—	N.D.	N.D.	—
			Secondary effluent	92[c]	2.2	161	175	385	208	284	625

Note—N.D. = not detectable (response at any sample dose level was less than 2-fold above concurrent solvent control).

[a] Refers to the concentration of extractable organics based on the residue weights of the extracts.

[b] Based on the mutagenicity values from the assays with S9.

[c] Extraction at pH 11.0 was omitted.

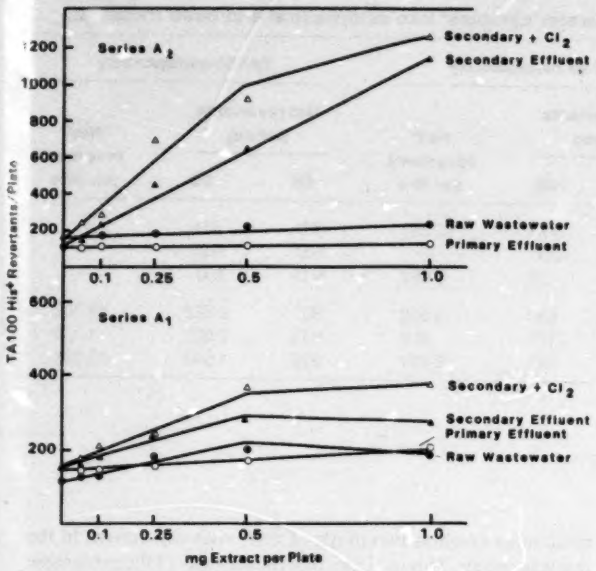

Figure 6—Mutagenic activity of organic extracts of wastewater samples collected at the Mill Creek T&E facility for *S. typhimurium* strain TA100 in the presence of metabolic activation.

of mutation induction. The results (Figure 7) show clear evidence of mutagenic activity for strains TA98, TA100, and TA1537. Strain TA98 was the most sensitive and most responsive of the strains to the mutagens present in this sample. Strain TA98 generally exhibited higher sensitivity and responsiveness in comparison to strain TA100 for the other samples tested as well (Table 2).

The influence of S9 on mutagenicity levels of the extracts varied greatly between samples. For every sample examined, direct-acting (non-S9 dependent) mutagens were present. However, for some samples (Series A_1, secondary effluent) activity levels were substantially increased with the addition of S9. S9 also seemed to reduce the toxicity of the samples as indicated by the fact that linear increases in the dose-response curves generally occurred up to higher dose levels, as opposed to assay results without S9 (Figures 3–7). In general, mutagenicity was considered not detectable in more of the extracts tested without S9 (Table 2), especially with TA100. Increases two-fold above background were never reached despite evidence in many cases for increasing response at lower doses.

To compare the total mutagenic activities associated with the different wastewaters and overall removal with treatment, the number of revertants were calculated in terms of litre equivalents of the original wastewaters. The concentrations of extractable residue organics were multiplied by the specific mutagenicities of the residues in the extracts. For comparison of activities, the data with strain TA98 plus S9 seems most appropriate because the highest responses were generally obtained using this assay condition and because positive responses were consistently detectable at different treatment stages. On the basis of these data, the overall removal of mutagenic activity during treatment varied among test series from no removal to a reduction of as much as two-thirds of the activity. In fact, this variability in removal occurred at the T&E pilot plant with samples taken only 1 week apart. It is notable that the mutagenicity levels in a single test series of a domestic wastewater effluent (Muddy Creek Plant) were substantially lower (7- to 45-fold) than any of the test series taken at the Mill Creek Plant and T&E Facility.

For the last test series collected, the association of mutagenic activity with acid/neutral and basic compounds was examined (Table 3). The percentage of the total extractable organic residue in the acid/neutral fraction was 90% for the primary effluent and 94% for the secondary effluent. The only detectable mutagenic activity in the primary effluent was found in the acid/neutral fraction. For the secondary effluent, the highest specific mutagenic activity was associated with the base fraction. However, because of the small percentage of the total weight contributed by the basic components, the overall mutagenic activity attributable to basic compounds was minor. The data in Table

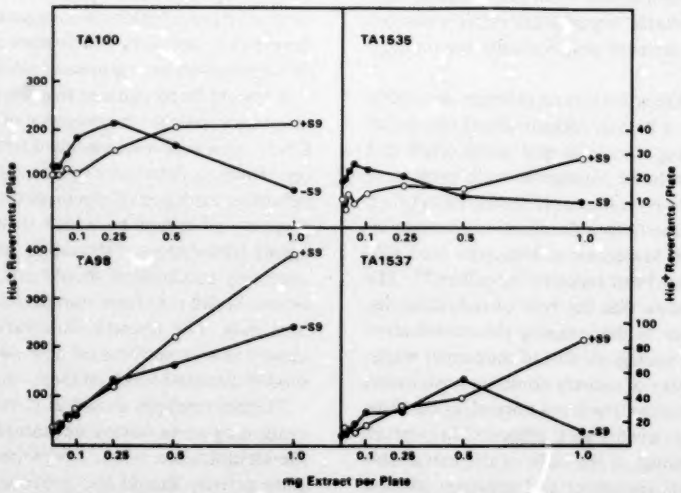

Figure 7—Strain specificity of mutation induction with an organic extract of a secondary effluent sample collected from the Mill Creek plant (Series B_1).

Table 3—Distribution of mutagenic activity of municipal wastewater samples[a] into acid/neutral and base fractions.

Treatment stage	Fraction	Concentration[b] (mg/L)	TA98 mutagenicity: Net revertants per mg −S9	TA98 mutagenicity: Net revertants per mg +S9	TA98 mutagenicity: Net[c] revertants per litre	TA100 mutagenicity: Net revertants per mg −S9	TA100 mutagenicity: Net revertants per mg +S9	TA100 mutagenicity: Net[c] revertant per litre
Primary effluent	Acid/neutral	63.5	N.D.	174	12 250	N.D.	N.D.	—
	Base	6.8	N.D.	N.D.	—	N.D.	N.D.	—
	Combined fractions	70.4	N.D.	129	9 082	N.D.	N.D.	—
Secondary effluent	Acid/neutral	8.2	466	650	5 556	907	2 222	18 309
	Base	0.4	1 264	1 171	497	N.D.	2 667	1 132
	Combined fractions	8.7	693	761	6 621	928	1 540	13 398

Note—not detectable.

[a] Sample series B_2, collected 6-20-83.

[b] Concentration of extractable organics based on residue weights of the extracts.

[c] Based on the mutagenicity values from the assays with S9.

3 also indicate that the TA98 mutagenicity of the two fractions of the secondary effluent, when tested individually, were close to being additive based on the activity of an extract prepared by combining the fractions in the appropriate proportions. However, some antagonism among the components in the two fractions was apparent for the TA100 +S9 mutagenicity for the secondary effluent and also for the TA98 mutagenicity for the primary effluent.

DISCUSSION AND CONCLUSIONS

In complex mixtures such as municipal wastewaters, metals and inorganic pollutants with potential health effects can generally be identified by standard specific analyses and their effects estimated directly. In addition, the volatile organics are also relatively easily separated and individually identified and tend not to persist in surface waters. Thus, the extractable and nonextractable semivolatile and nonvolatile organics are the classes of pollutants where health effects screening assays of wastewater effluents discharged into surface waters seem most appropriate. Of the two classes, the extractable organics are easily separated and concentrated and were selected pragmatically for mutagenicity evaluation.

Mutagenic activity in the Ames test was consistently detectable in extracts of effluents from a heavily industrialized municipal wastewater. Both direct-acting mutagens and compounds that require metabolic activation to be mutagenic were present. It seems that the mutagenic activity is primarily industrial in origin because the level of mutagenicity in a domestic wastewater effluent was substantially lower. Mutagenic activity associated with totally industrial effluents has been reported by others.[4,12] The studies by McGeorge[12] indicate that the type of industrial discharge is an important factor in determining the contribution of industrial effluents to the mutagenicities of municipal wastewaters. Clearly, a larger number of entirely domestic wastewaters need to be examined to determine whether a normal, or baseline, level of mutagenic activity exists for such effluents. In contrast to consistently efficient removal of the bulk of the extractable organics during conventional treatment and similarly efficient removal of many priority pollutant chemicals reported elsewhere,[9] removal of mutagenic activity varied from essentially none to as much as two-thirds of that originally present in the raw wastewater. This indicates that the majority of the extractable mutagenic activity in raw wastewater is associated with a small fraction of the total organics present. This result also suggests that an appreciable portion of the responsible mutagens are relatively refractory to removal by conventional primary and activated sludge treatment.

Chlorination of the secondary effluent did not have a substantial impact on the mutagenicity of the two effluents examined. This agrees with the studies of Johnston *et al.*[9] who did not detect any obvious influence of chlorination in their survey of effluents in Illinois. The apparent lack of a major influence of chlorination during wastewater treatment is in contrast to what is often found in studies of drinking water concentrates where a major part of the mutagencity has been found to arise during the chlorination stage of water treatment,[13] most likely as a result of reaction with humic substances in the source waters.[14] One explanation for this discrepancy is that the high levels of ammonia in wastewater compete for the chlorine, which results in high levels of inorganic chloramine formation relative to production of chlorinated organics. Further work needs to be done before any firm conclusions are reached about the effects of chlorination on wastewater mutagenicity.

It should be recognized that the mutagenic responses in bacterial tests indicate the presence of chemicals that interact with DNA. Such action is associated with chemicals that induce cancer. However, detection of such activity does not, in itself, provide definitive evidence of carcinogenic risk. The intact animal has a variety of means by which it modifies its response to such agents (absorption, distribution, metabolism, excretion). Consequently conclusions should not be drawn concerning quantitative health risks from mutagenic activity based solely on Ames Test data. The research observations in this study should be viewed as a perspective on how wastewater treatment processes modify detected levels of these mutagens.

Further research aimed at strengthening this perspective by examining more wastewater samples is needed. Efforts towards the identification of the compounds responsible for the mutagenic activity should also prove important, both to help determine the source of the mutagens and to devise effective treatment methods for their removal.

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